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Patent
Attorney's Docket No. 000270-007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

COPY

In re Patent Application of	
Steven L. STICE et al	Group Art Unit: 1819
Serial No.: 08/781,752	Examiner: D. Crouch
Filed: January 10, 1997	
For: CLONING USING DONOR NUCLEI FROM DIFFERENTIATED FETAL AND ADULT CELLS	

DECLARATION OF JAMES M. ROBL Ph.D. PURSUANT TO 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, James M. Robl, Ph.D., declare and state as follows:

(1) I reside at 196 Old Enfield, Belchertown, Massachusetts 01007;

(2) I am a Professor in the Veterinary & Animal Sciences Department at the University of Massachusetts at Amherst Massachusetts;

(3) I have substantial knowledge and expertise in the areas of cloning and transgenic animals. My expertise is substantiated by the attached curriculum vitae.

(4) Based on such expertise, I am frequently asked to give presentations and talks relating to cloning and transgenic animals.

(5) I have reviewed the most recent Office Action issued on June 22, 1998, in the above-identified application. In particular, I have carefully reviewed the enablement rejection that bridges pages 5 to 8 of the Office Action. Based on such review, it is my understanding that the Examiner apparently is of the opinion that the subject application only enables a method for cloning a bovine comprising the specific steps of:

(i) inserting the fibroblast or nucleus isolated from a 45 day bovine fetus into the perivitelline space of a bovine oocyte matured *in vitro* to metaphase II;

(ii) fusing the oocyte and fibroblast or nucleus to form a NT unit; activating the NT unit by incubating such NT unit for 26-27 hours post-maturation in media comprising 5 um ionomycin and 2mM DMAP for 4 minutes;

(iii) culturing the NT units in CR1aa-2mM DMAP media for 4-5 hours;

(iv) and culturing the activated NT units in CR1aa media containing mouse fibroblast feeder cells for 5-8 days after activation and transforming to a host bovine for development into a fetus; as well as offspring and progeny produced by such methods; and producing a bovine CICM cell by the same method except that the transfer to a host bovine is omitted and the cultured activated NT units are

desegregated to produce the inner cell mass of embryos. Based on the following, I respectfully disagree with the Examiner's conclusion.

(6) However, prior to specifically addressing the enablement concerns raised in the office Action, I will summarize some of the novel discoveries which form the basis of the subject invention. It is believed that this discussion will clarify why I am of the opinion that the claims are commensurate in scope with the subject disclosure, especially given the truly pioneering nature of the present invention.

(7) In particular, the novel developments discovered by the inventors of this application include:

i) the successful use of cells committed to a somatic cell lineage for nuclear transfer or transplantation;

ii) the successful use of actively dividing, i.e., non-quiescent cells for nuclear transplantation; and

iii) the use of somatic cell genetic modification to produce genetically modified animals.

Prior to the filing date of this application, to the best of my knowledge, there had been no previous report of the use of cells committed to a somatic cell lineage for successful nuclear transplantation, i.e., that gave rise to viable offspring. I believe this

to be a novel and surprising discovery based on the history of nuclear transplantation prior to the present invention.

(8) In this regard, I note that nuclear transfer first gained acceptance in the 1960's with amphibian nuclear transplantation. (Diberardino, M.A. 1980, "Genetic stability and modulation of metazoan nuclei transplanted into eggs and ooctyes", *Differentiation*, 17-17-30; Diberardino, M.A., N.J. Hoffner and L.D. Etkin, 1984; "Activation of dormant genes in specialized cells", *Science*, 224:946-952; Prather, R.S. and Robl, J. M., 1991, "Cloning by nuclear transfer and splitting in laboratory and domestic animal embryos", In: *Animal Applications of Research in Mammalian Development*, R.A. Pederson, A. McLaren and N. First (ed.), Spring Harbor Laboratory Press.) Nuclear transfer was initially conducted in amphibians in part because of the relatively large size of the amphibian oocyte relative to that of mammals. The results of these experiments indicated to those skilled in the art that the degree of differentiation of the donor nucleus was greatly instrumental, if not determinative, as to whether a recipient oocyte containing such cell or nucleus could effectively reprogram said nucleus and produce a viable embryo. (Diberardino, M.A., N.J. Hoffner and L.D. Etkin, 1984, "Activation of dormant genes in specialized cells.", *Scienc,e* 224:946-952; Prather, R.S. and Robl, J. M.,1991, "Cloning by nuclear transfer and splitting in laboratory and domestic animal embryos", In: *Animal Applications of*

Research in Mammalian Development, R.A. Pederson, A. McLaren and N. First (ed.), Spring Harbor Laboratory Press) This work is well documented and was considered dogma prior to the filing of this application.

(9) Much later, in the mid 1980's, after microsurgical techniques had been perfected, researchers, including myself, investigated whether nuclear transfer could be extrapolated to mammals. I worked out the first procedures for cloning cattle (Robl, J. M., R. Prather, F. Barnes, W. Eyestone, D. Northey, B. Gilligan and N.L. First, 1987, "Nuclear transplantation in bovine embryos", *J. Anim. Sci.*, 64:642-647) and my lab was the first to clone a rabbit by nuclear transfer using donor nuclei from earlier embryonic cells (Stice, S.L. and Robl, J. M., 1988, "Nuclear reprogramming in nuclear transplant rabbit embryos", *Biol. Reprod.*, 39:657-664). Also, using similar techniques, bovines (Prather, R.S., FL. Barnes, ML. Sims, Robl, J. M., W.H. Eyestone and N.L. First, 1987, "Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte", *Biol. Reprod.*, 37:859-866) and sheep (Willadsen, S.M., 1986, "Nuclear transplantation in sheep embryos", *Nature*, (Lond) 320:63-65), and putatively porcines (however, this work apparently has never been reproduced) (Prather, R.S., M.M. Sims and N.L. First, 1989, "Nuclear transplantation in pig embryos", *Biol. Reprod.*, 41:414), were cloned by the transplantation of the cell or nucleus of very early embryos into enucleated oocytes.

(10) Moreover, work in our laboratory, and others, investigated the possibility of producing nuclear transfer embryos with donor nuclei obtained from progressively more differentiated cells. However, our results and those obtained by other groups, indicated that when the embryo progresses to the blastocyst stage (the embryonic stage where the first two cell lineages separate) that the efficiency of nuclear transfer decreases dramatically (Collas, P. and J.M. Robl, 1991, "Relationship between nuclear remodeling and development in nuclear transplant rabbit embryos", *Biol. Reprod.*, 45:455-465). For example, it was found that trophectodermal cells (the cells that form the placenta) did not support development of the nuclear fusion to the blastocyst stage. (Collas, P. and J.M. Robl, 1991, "Relationship between nuclear remodeling and development in nuclear transplant rabbit embryos", *Biol. Reprod.*, 45:455-465) By contrast, inner cell mass cells (cells which form both somatic and germ cells) were found to support a low rate of development to the blastocyst stage with some offspring obtained. (Collas P, Barnes FL, "Nuclear transplantation by microinjection of inner cell mass and granulosa cell nuclei", *Mol Reprod Devel.*, 1994, 38:264-267) Moreover, further work suggested that inner cell mass cells which were cultured for a short period of time could support the development to term. (Sims M, First NL, "Production of calves by transfer of nuclei from cultured inner cell mass cells", *Proc Natl Acad Sci* ,1994, 91:6143-6147)

(11) Based on these results, and that of other researchers, it was the overwhelming opinion of those skilled in the art at that time, including myself, that observations made with amphibian nuclear transfer experiments would likely be observed in mammals. That is to say, it was widely thought by researchers working in the area of cloning prior to the present invention that once a cell becomes committed to a particular somatic cell lineage that its nucleus irreversibly loses its ability to become "reprogrammed", i.e., to support full term development when used as a nuclear donor for nuclear transfer. While the exact molecular explanation for the apparent inability of somatic cells to be effectively reprogrammed was unknown, it was hypothesized to be the result of changes in DNA methylation, histone acetylation and factors controlling transitions in chromatin structure that occur during cell differentiation. Moreover, it was believed that these cellular changes could not be reversed.

(12) The discovery made by the present inventors, and subsequently reported by the Roslin Institute after the filing date of this application, i.e., that cells committed to somatic cell lineage could support development when used as nuclear transfer donors, is actually the culmination of a progression of experiments and observations made by our laboratory. For example, we demonstrated in 1990 (unpublished observations, Collas, P. and J.M. Robl, 1991, "Relationship between nuclear

remodeling and development in nuclear transplant rabbit embryos", *Biol. Reprod.*, 45:455-465; Collas P, Barnes FL, "Nuclear transplantation by microinjection of inner cell mass and granulosa cell nuclei", *Mol Reprod Devel*, 1994, 38:264-267) that somatic cells could support development to the blastocyst stage, but not beyond. This seemingly confirmed the general view held by those skilled in the cloning art at that time concerning the irreversible changes to cells that occur during differentiation. In fact, because of this erroneous belief, our first work with somatic cell nuclear transplantation was not conducted with the goal of producing full term offspring. Our laboratory, like others at that time, was of the opinion that this would not be feasible. Rather, we were interested in producing blastocysts by somatic cell nuclear transplantation, and using the resultant blastocyst stage embryos to produce ES-like cells. The thought was that somatic cell-derived ES-like cells might be able to contribute to the development of fully differentiated tissues if grown in association with normal cells in a chimera. This work was successful. (Cibelli, J.B., S.L. Stice, P.J. Golueke, J.J. Kane, J. Jerry, C. Blackwell, F.A. Ponce de Leon and Robl, J.M., 1998, "Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells", *Nature/Biotechnology*,16: 642-646)

(13) Based on the surprising success of these experiments, we tried to determine whether somatic cell nuclear transplant embryos could support early

development on their own *in vivo* and provide a source of fetal tissue. These experiments were also surprisingly effective and resulted in healthy 40 day fetuses. (Zwada, M.W., J.B. Cibelli, PK. Choi, E.D. Clarkson, P.J. Golueke, S.E. Witta, K.P. Bell, J. Kane, F.A. Ponce de Leon, D.J. Jerry, Robl, J.M., C.R. Freed and S.L. Stice, 1998, "Somatic cell cloning-produced transgenic bovine neurons for transplantation in parkinsonian rats", *Nature Medicine*, 4:569-574)

(14) In the course of these studies we also determined whether these fetal cells could be genetically manipulated *in vitro*, i.e., by the introduction of a heterologous DNA by electroporation, prior to their use as nuclear transplant donors. These experiments were effected because an important goal of cloning, if not the most important goal of cloning, is to provide a reproducible source of cells having a desired genotype, e.g., which express a particular transgene. Thereby, the resultant cloned embryos or animals can be used to produce a desired gene product or for cellular transplantation therapies. However, it was by no means predictable that these experiments would be successful. To the contrary, I am of the opinion, based on the state of the art at the time of the invention, that the prevailing expectation would have been that these cells, given their differentiated state, coupled with the fact that they were manipulated in tissue culture and then transfected with a foreign DNA, would either not give rise to nuclear transfer embryos at all, or would produce embryos that

would only differentiate to early stages. However, as can be seen from the experiments, the results of which are reported in this application, quite surprisingly, these cells when inserted in enucleated bovine oocytes gave rise to apparently perfectly healthy 40 day transgenic bovine embryos.

(15) Thereupon, based on the surprising success of the above-described experiments, we then attempted to determine whether somatic cell nuclear transplant embryos would give rise to viable full-term bovine offspring, and more desirably, transgenic viable full-term bovine offspring. As the Examiner is aware, and has been well reported in the press, it was astoundingly discovered that cells which are committed to a differentiated cell type, which cells were moreover made transgenic (transgenic fetal fibroblasts) when used as nuclear transfer donors, gave rise to healthy, transgenic bovine offspring. Moreover, these results have been successfully repeated by us and other groups. In fact, based on the reproducibility and efficiency of the subject cloning technique, the licensee of this application has entered into a collaboration with Genzyme Transgenics Corporation to make cloned transgenic bovines that produce a polypeptide (HSA) in their milk. It is further noted that using the basic cloning methods which are the subject of this application, a transgenic bovine that contains the HSA gene has been successfully obtained. With this basic understanding of the state of the nuclear transfer art that existed prior to the present

invention, and the developments and discoveries that culminated in the subject invention, I will now address the various enablement concerns raised by the Examiner.

(16) Types and Age of Differentiated Cells Used for Nuclear Transfer

As discussed, our invention involves the generic discovery that cells committed to a somatic cell lineage, which optionally are transgenic, can be used as nuclear transfer donors to produce viable fetuses and offspring. Contrary to the Examiner's position, the efficacy of the invention does not require that such somatic cells be fibroblasts isolated from 45 day old bovine offspring. In fact, our results and those of others supports our claim that a wide variety of types and ages of cells committed to a somatic cell lineage can be used successfully for nuclear transfer. Moreover, our results and those of others further support the view that these cells may be used to produce cloned transgenic animals.

(17) For example, two calves have been produced at the Ishikawa Prefecture Livestock Research Centre in Japan from oviduct cells collected from a cow at slaughter. (Hadfield, P. and A. Coghlan, "Permature birth repeats the Dolly mixture", *New Scientist*, July 11, 1998) Also, Jean-Paul Renard from INRA in France has produced a calf from muscle cells from a fetus. (MacKenzie, D. and P. Cohen, 1998., "A French calf answers some of the questions about cloning", *New Scientist*, March 21) Further, David Wells from New Zealand has produced a calf from fibroblast cells

from an adult cow. (Wells, D.N., 1998, "Cloning symposium: Reprogramming Cell Fate - Transgenesis and Cloning", Monash Medical Center, Melbourne, Australia, April 15-16)

(18) Moreover, our experiments to date indicate that cells obtained from fetuses, calves, young adults and aged adults all can be grown in culture and can be used as nuclear donor cells to produce cloned animals. However, it should be noted that the length of the cell cycle and the life-span of the cells shortens with the age of the donor. Furthermore, the percentage of healthy embryos produced from adult cells decreases with the age of the animal. This suggests that actively dividing cells can more readily support development following nuclear transfer than cells progressing towards a quiescent state. However, it does not support a proposition that only cells of a certain age can be used as suitable nuclear transfer donors.

(19) Interestingly, there is considerable variation in the length of the cell cycle within these populations. In fetal populations, some cells divide as rapidly as every 12 hours with most having divided by 24 hours. By contrast, in cell populations obtained from adult cells, a few cells will divide relatively quickly, but most require more than 24 hours to divide. These results explain why adults can be cloned, but the efficiency appears to be much lower than with fetuses. These observations also lend credence to the observation made by us, namely that actively dividing cells are capable

of being used as donor nuclei, or cells, during nuclear transfer to produce viable embryos.

This is contrary to the work of the Roslin Institute (published after the filing date of this application), which instead reported the use of quiescent, i.e., non-actively dividing cells, to produce cloned sheep. (Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS, "Viable offspring derived from fetal and adult mammalian cells", *Nature*, 1997, 1385:810-813) In fact, this significant difference in our cloning method *vis-a-vis* that reported by the Roslin Institute was discussed by us in a letter to the Editors of *Science*, 281:1611 (1998) (a copy of which is attached to this Declaration). In my opinion, this difference explains why the cloning methods reported in this application are highly reproducible.

(20) Non-Criticality of *In vitro* Maturation

The Examiner has seemingly concluded that the use of *in vitro* matured oocytes is critical to the efficacy of the invention. This, however, is not correct. While the Examiner is correct in the fact that the use of *in vitro* matured oocytes is exemplified in the actual working examples, this is not essential to the efficacy of the subject cloning methods. *In vitro* matured oocytes were used largely because of supply and cost concerns. Essentially, immature oocytes, rather than *in vivo* matured oocytes were used because we did not have access to *in vivo* matured oocytes and because *in vitro*

mature oocytes can be obtained in plentiful numbers (e.g., from slaughterhouse suppliers). However, as stated in our patent application, *in vivo* matured oocytes can also be used for nuclear transfer. For example, *in vivo* matured oocytes can be collected from either non-superovulated or ovulated cows or heifers and used for enucleation. In fact, *in vivo* oocytes have recently been used by us for cloning and a calf was successfully produced this month. Therefore, it is clear that the efficacy of the subject cloning method does not rely on a particular *in vitro* matured procedure, or even that *in vitro* matured oocytes be used at all.

(21) Oocyte Activation Protocol

Also, contrary to the Office Action, the efficacy of the subject cloning methods is not limited to the specific activation conditions exemplified in the working examples. As explained above, the truly novel discovery made by us are that (1) cells committed to a somatic cell lineage can support development to term following nuclear transfer; (2) the use of actively dividing, non-quiescent, cells for nuclear transplantation, and (3) the use of somatic cell genetic modification to produce genetically modified animals. The specific activation protocol that is used was not critical to the cloning method.

(22) With respect thereto, my laboratory has studied the activation process in great detail over the past twelve years. Activation is a process that involves the

elevation of intracellular calcium in the egg. The sperm normally produces oscillations in calcium concentration that last for several hours. Artificial activation protocols have been used on eggs for many years. Early work indicated that ethanol, electrical shock, cooling, calcium-free media, various anesthetics and a variety of other stimuli could cause activation. (Whittingham, D.G., 1980, "Parthenogenesis in mammals", *Oxford Rev. Reprod, Biol.*, 2:205-231) In more recent years, with the development of procedures for measuring intracellular calcium and the various intracellular responses to calcium, more specific approaches have been developed. For example, we now know that electrical pulses cause transient increases in intracellular calcium by inducing pores in the membrane and allowing calcium to flood into the cell from the extracellular media. (Fissore, R.A. and Robl, J.M., 1992, "Intracellular calcium response of rabbit oocytes to electrical stimulation", *Mol. Reprod. Devel.*, 32:9-16; Collas, P., J.J. Balise, G.A. Hofman and Robl, J.M., 1989, "Electrical activation of mouse oocytes", *Theriogenology*, 32:835-844; Robl, J.M., P. Collas, R. Fissore and JR. Dobrinsky, 1992, "Electrically induced fusion and activation in nuclear transplant embryos", In: *Guide to Electroporation and Electrofusion*; D. Chang, B.M. Chassy, J.A. Saunders and A.E. Sowers (ed.), Academic Press, Inc., San Diego, CA; Collas, P., R. Fissore, J. M. Robl, E.J. Sullivan and F.L. Barnes, 1993, "Electrically-induced calcium elevation, activation and parthenogenetic development of bovine oocytes", *Mol. Reprod. Devel.*,

34:212-223; Collas, P., R. Fissore and J.M. Robl, 1993, "Preparation of nuclear transplant embryos by electroporation", *Anal. Biochem.*, 208:1-9) Multiple pulses can be used to duplicate sperm-induced calcium oscillations. Injection of such intracellular second messengers such as IP3, or its long acting analogues, GTP, or its long acting analogues, or calcium itself can duplicate sperm-induced calcium rises. Other compounds that cause calcium rises, although less physiological, are ethanol and calcium ionophores. (Fissore, R.A. and Robl, J.M., 1993, "Sperm, inositol trisphosphate and thimerosal induced intracellular Ca2+ elevations in rabbit eggs", *Devel. Biol.*, 159:122-130; Fissore, R.A. and Robl, J.M., 1994, "Mechanism of calcium oscillations in fertilized rabbits eggs", *Devel. Biol.*, 166:634-642; Fissore, R.A., Pinto-Correia, C. and J.M. Robl, 1995, "Inositol trisphosphate-induced calcium release in the generation of calcium oscillations in bovine eggs", *Biol Reprod.*, 53:766-774; Collas, P., Chang, T., Long, C. and J.M. Robl, 1995, "Inactivation of histone H1 kinase by Ca^{2+} in rabbit oocytes", *Mol. Reprod. Devel.*, 40:253-258.) The second part of the activation event is a decrease in a cell cycle regulatory kinase called MPF. This results in a decrease in the phosphorylation of many different proteins in the cell and the progression to interphase. This part of the process can be duplicated by various kinase inhibitors. MPF can be inactivated directly by inhibiting protein synthesis and compounds like puromycin and cycloheximide have

been used successfully in oocyte activation protocols. Currently, there are a number of different combinations of the above that are being used successfully for oocyte activation in various laboratories around the world. Our use of the ionomycin/DMAP procedure was more a matter of convenience than a view that it was better than various other protocols, and it is certainly not critical for the success of the procedure.

For the Examiner's convenience, a brief overview of different activation procedures known prior to the filing of this application are summarized below:

Cell type	Activation	Culture media	Reference
Blastomeres	Electrical	B2 + oviductal cells	(Ectors et al. 1995)
Blastomeres	Electrical	CRI	(Zakhartchenko et al. 1995)
Blastomeres	Electrical	Bovine oviduct epithelial cells	(Campbell et al. 1993)
Blastomeres	Electrical	Modified Brinsters Ovum Culture Medium	(Barnes et al. 1993)
Blastomeres	Chilling	Sheep oviduct	(Westhusin et al. 1996)
ICM cells	Chilling	CRI + MEM + BME	(Keefer et al. 1994)
Oogonia	Ion+DMAP	TCM 199 + Steer serum	(Lavoir et al. 1997)
Blatomeres	Electrical	TCM 199 + calf serum	(Takano H. 1996)

Barnes, F. L., Collas, P., Powell, R., King, W. A., Westhusin, M., and Shepherd, D. (1993). "Influence of Recipient Oocyte Cell Cycle Stage on DNA Synthesis, Nuclear Envelope Breakdown, Chromosome Constitution, and Development in Nuclear Transplant Bovine Embryos." *Molecular Reproduction and Development*(36), 33-41.

Campbell, K. H. S., Ritchie, W. A., and Wilmut, I. (1993). "Nuclear-Cytoplasmic Interactions during the First Cell Cycle of Nuclear Transfer Reconstructed Bovine Embryos: Implications for Deoxyribonucleic Acid Replication and Development." *Biology of Reproduction*(49), 933-942.

Ectors, F.J., Delval, A., Smith, L. C., Touati, K., Remy, B., Beckers, J.-F., and Ectors, F. (1995). "Viability of Cloned Bovine Embryos After One or Two Cycles of Nuclear Transfer and In Vitro Culture." *Theriogenology*(44), 925-933.

Keefer, C. L., Stice, S. L., and Matthews, D. L. (I 994). "Bovine Inner Cell Mass Cells as Donor Nuclei in the Production of Nuclear Transfer Embryos and Calves." *Biology of Reproduction*(50), 935-939.

Lavoir, M.-C., Rumph, N., Moens, A., King, W. A., Plante, Y., Johnson, W. H., Ding, J., and Betteridge, K. J. (I 997). "Development of Bovine Nuclear Transfer Embryos Made with Oogonia." *Biology of Reproduction*(56), 194-199.

Takano H., K. C., Kato Y, Tsunoda Y. (I 996). "Cloning of bovine embryos by multiple nuclear transfer." *Theriogenology*, 47, 1365-1373.

Westhusin, M. E., Collas, P., Marek, D., Sullivan, E., Stepp, P., Pryor, J., and Barnes, F. (1996). "Reducing the Amount of Cytoplasm Available for Early Embryonic Development Decreases the Quality But Not Quantity of Embryos Produced by In Vitro Fertilization and Nuclear Transplantation." *Theriogenology*(46), 243-252.

Zakhartchenko, V., Wolf, E., Palma, G. A., and Brem, G. (I 995). "Effect of Donor Embryo Cell Number and Cell Size on the Efficiency of Bovine Embyro Cloning." *Molecular Reproduction and Development*(42), 53-57.

(23) Culture Medium Used to Maintain Nuclear Transfer Embryos

Also, contrary to the Office Action, a particular culture medium is not critical to the efficacy of the invention. Indeed, there are many different media that can be used interchangeably for growing bovine embryos in culture. The only important factor with

respect to such culture media is that they be based on a Krebbs-Ringer bicarbonate solution. This solution has a variety of salts and a bicarbonate buffer system that is based on the analysis of blood serum components. Such media generally contain an energy source, antibiotics and usually some complex component such as serum or co-cultured cells. In use currently for culture of bovine embryos are simple media, complex media, co-culture systems with cumulus cells, BRL cells or fibroblast cells, and completely defined media. (Different method in current usage can also be found in the Table in paragraph (22) supra.) Moreover, in my laboratory, one student is using a complex media with a cumulus cell co-culture while another student is using a simple media with a fibroblast co-culture. Both students are doing nuclear transplantation work and having success with development of embryos. The reason for using the different culture systems in the same lab simply is that one student is more comfortable with one system, and the other is more comfortable with the other system. Therefore, it is apparent that a specific culture media is not essential to the efficacy of the subject invention.

(24) Use of Method for Produce Other Cloned Species (Non-Bovines)

In my expert opinion, the subject cloning methods can be used to clone different mammals, i.e., other than bovines. That is to say, I am of the opinion that the basic discovery made by us that cells committed to a somatic lineage, preferably non-

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quiescent cells, can be used as nuclear transfer donors to produce nuclear transfer embryos that give rise to viable fetuses and offspring, can be extrapolated to different, i.e., non-bovine mammals. In fact, subsequent to the filing of this patent application, I am aware that differentiated cells have reportedly been successfully used to produce cloned sheep and mice. (Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS, "Viable offspring derived from fetal and adult mammalian cells", *Nature,* 1997, 1385:810-813; Wakayama T, Perry ACF, Zucconi M, Johnsoal KR, Yanagimachi R., "Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei", *Nature,* 1998, 394:369-374)

(25) The generic nature of our discovery is further supported by recently reported experiments conducted by our lab relating to the efficacy of cross-species nuclear transplantation. Specifically, we conducted an experiment wherein we successfully produced a nuclear transfer fusion embryo by the insertion of an adult differentiated cell (obtained from the cheek of an adult human donor) into an enucleated bovine oocyte. This further substantiates the fact that adult differentiated cells of different species can be successfully "reprogrammed", notwithstanding the previous dogma that existed prior to the present invention, i.e., the widely-held but mistaken belief that only very early non-differentiated cells could be used for nuclear transfer donor cells or nuclei.

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(26) In summation, I hope that I have adequately addressed all of the Examiner's enablement concerns. For the reasons set forth above, and those enumerated in the present application, I am of the opinion that the efficacy of the subject invention does not depend upon the specific parameters, e.g., cell type, age, *in vitro* maturation, oocyte activation method, utilized in our working examples. Moreover, I believe that the Patent Office's conclusion is further untenable given the truly pioneering nature of the invention and further based on the fact that subsequent to the invention, differentiated cells have reportedly been successfully used by numerous groups to produce nuclear transfer embryos and cloned offspring.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 12/9/98 James M. Robl

James M. Robl, Ph.D.